METHOD FOR PRODUCING BIOLOGICALLY ACTIVE HUMAN FACTOR VIII IN THE MILK OF TRANSGENIC ANIMALS DRIVEN BY MAMMARY-SPECIFIC EXPRESSION CASSETTES

BACKGROUND OF THE INVENTION

1. Field of the invention

The present invention discloses a method for producing biologically active recombinant proteins in the milk of transgenic animals, characterized in which intact human clotting factor VIII gene and B-domain deleted recombinant factor VIII gene are transferred into the mammal by gene microinjection and embryonic implantation to obtain expression and secretion in the milk of transgenic animals and their offsprings.

2. Description of the prior art

Hemophilia is the most common human bleeding disorder and affects approximately one in 5,000 males, causing lifelong, repeated, and potentially life-threatening hemorrhagic episodes. It results from a deficiency in functional blood coagulation factors, either in a serine protease called Factor IX (hemophilia B) or in its cofactor Factor VIII (hemophilia A). Because hemophilia is caused by defects in single X-linked genes that encode circulating plasma proteins, the development of therapeutic strategies has focused on finding ways to replace the defective or deficient proteins.

Human FVIII is synthesized primarily in the liver and secreted into the circulation at low plasma concentration, approximately 100-200 ng/ml. Analysis of the cloned cDNA for human FVIII (Gitschier et al., 1984, Nature 312: 326-330;

Toole et al., 1984, Nature 312: 342-347.) has provided more information about its gene and protein structure. The full length FVIII cDNA contains an open reading frame coding for a polypeptide of 2351 amino acids. This primary translation product contains a 19 a.a. signal peptides and six homologous domains in the order A1-A2-B-A3-C1-C2 (Vehar et al., 1984, Nature 312: 337-342.). The 256-kDa precursor protein is proteolytically processed intracellularly to a metal ion-linked heterodimer of a 90- to 200-kDa heavy chain (A1-A2-B) and an 80-kDa light chain (A3-C1-C2), which circulates in the plasma bound to the von Willebrand factor (vWF).

For the past two decades hemophiliacs have been treated with whole plasma, and more recently with highly purified FVIII concentrates prepared from plasma. Higher concentrates purity allows the administration of effective doses at lower volumes to patients. This therapy is effective in controlling bleeding episodes, however, hemophiliacs may suffer from other complications, compromised by the discovery that these products were transmitting human viruses such as the hepatitis B virus and human immuno-deficiency virus (HIV) to patients, many of them later developed diseases (Hoyer, 1993, Methods Enzymol. 222: 169-176).

Since then, plasma screening and viral inactivation procedures have greatly improved the safety of these products, although the potential for transmitting diseases such as Creutzfeldt-Jakob disease must be considered and has led to sporadic shortages in these plasma products. Nonetheless, when non-blood source products derived from recombinant DNA biotechnology became available, further reducing the viral risk, they were quickly embraced by caregivers and patients, despite their higher cost (Pipe and Kaufman, 2000, Nature Biotech. 18: 264-265).

One of the challenges created by the biotechnology revolution is the development of methods for the economical production of highly purified proteins in large scales. Recent developments indicate that manipulating milk composition using transgenesis has focused mainly on the mammary gland as a bioreactor to produce pharmaceuticals.

SUMMARY OF THE INVENTION

The ability to modify animal genomes through microinjection technology has offered new alternatives for the manufacture of recombinant proteins. Targeting the production of human recombinant protein pharmaceuticals in the milk of transgenic animals solved many problems associated with either microbial or animal cell expression systems. Bacteria often improperly fold complex proteins, introducing more involved and expensive processes. Both bacteria and yeast lack adequate post-translational modification. Bioreactors for cell cultures require high capital expenditures, use large volumes of expensive culture media, and often suffer from relatively low yields.

To express a recombinant protein in the milk of a transgenic animal, expression vectors containing a gene encoding the protein of interest fused to milk specific regulatory promoter elements are generally introduced by microinjection into a pronuclear-stage embryo, or alternatively transfecting the expression vector into a cell line suitable for somatic cell nuclear transfer. Most of this work has been carried out with: the ovine β-lactoglobulin, rodent (mouse, rat and rabbit) whey acid protein (WAP) genes, bovine α-lactalbumin and a-s1-casein genes, as well as the caprine β-gene.

Several blood proteins, hormones, and enzymes have been synthesized in

transgenic animals. Of these, human FVIII is probably the largest and most complex protein to be expressed. Analysis of the cloned cDNA for human FVIII has provided more information about its gene and protein structure. The full length FVIII cDNA contains an open reading frame coding for a large polypeptide of 2351 amino acids. The 256-kDa precursor hFVIII protein is proteolytically processed intracellularly to a metal ion-linked hetero-dimer of a 90- to 200-kDa heavy chain (A1-A2-B) and an 80-kDa light chain (A3-C1-C2), which circulates in the plasma bound to the von Willebrand factor (vWF). The B-domain, is encoded by a single large exon and is highly glycosylated, harboring 19 of the 25 N-linked glycosylation sites. The B-domain is released upon co-factor activation, so it is not necessary for clotting function.

According to the regulatory sequences employed, variable levels of expression for the recombinant human FVIII were observed. For example, using a construct containing 2.2-kb ovine β-lactoglobulin 5'-flanking sequences to drive the FVIII cDNA with the introns of the murine metallothionein I (β-Lac/hFVIII-MtI), only extremely low level (4-6 ng/ml) of expression was observed in transgenic sheep (Niemann et al., 1999. Transgenic Res 8: 237-247). Whereas the hFVIII cDNA, under the control of the 2.5-kb mouse WAP promoter, was shown to secrete up to 2.7 μg/ml in transgenic pig milk (Paleyanda et al., 1997. Nature Biotech 15: 971-975).

In this invention, we describe a novel recombination of promoter, leader sequence, coding sequences and poly A tail signaling sequence as a expression cassette to generate of transgenic animals expressing the fusion gene in the mammary gland and the presence of recombinant FVIII protein ranged from 7 to 50 μg/ml with over 35- to 200-fold higher than in normal human plasma, with

temporal and spatial expression profiles for clotting active rFVIII in the milk of transgenic animals in different stages of lactation.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig.1A~1D shows a schematic map of full-length hFVIII transgene construction and detection of transgene integrated patterns in transgenic animals.

Fig.1A The structure of αLA-hFVIII-bGHpA fusion genes are shown and a pair of primer sets for PCR screening and a 1.8-kb hFVIII kpnI-fragment as probe were designed franking the 5'-end and 3'-end of hFVIII cDNA, respectively.

Fig.1B Rapid screening positive transgenic mice founders by PCR amplification. NC represented a normal mouse DNA as a negative control.

Fig.1C Screening of positive transgenic goat founders and F1 offspring by PCR amplification. NC represented a normal non-transgenic goat DNA as a negative control.

Fig.1D Southern blot analysis of the integration patterns of transgene DNA in transgenic animals. Genomic DNA extracted from different founders of transgenic mice tails or transgenic goats' ear tissues were digested with BamHI and subjected to probe hybridization.

Fig.2A~2B represents the determination of hFVIII transgene copy numbers in 17 transgenic founders using slot-blot hybridization.

Fig.2A The relative positions of sample loading for standard controls and transgenic founders. The standards were made with calculated hFVIII plasmid DNA, 0.5 to 20 copy numbers, mixed with 10 ug of normal mouse genomic DNA. The lanes of transgenic founders were also slotted with 10 ug of undigested

genomic DNA. A kpnI fragment of hFVIII cDNA was used as a hybridization probe. Hybridization and wash was performed in high stringency condition (70℃) to avoid cross-hybridization. Result is shown in Fig.2B.

Fig.3A~3C provides a schematic map of B-domain deleted hFVIII transgene construction and detection of transgene junctional sequences.

Fig.3A The structure of αLA-CN-hFVIII(△B)-bGHpA fusion genes are shown and two pairs of primer sets for PCR screening and a 1.8-kb hFVIII kpnI-fragment as probe were designed franking the 5'-end, B-domain flanking region and 3'-end of hFVIII cDNA, respectively.

Fig.3B DNA sequencing confirmed the in-frame creation of the N-terminal αS1-casein signal peptide sequence and the 19-aa leader pepteide-removed hFVIII sequence.

Fig.3C DNA sequencing confirmed the in-frame creation of B-domain deletion between hFVIII A-domain (A1-A2) and hFVIII C-domain (A3-C1-C2).

Fig.4A~4C shows the analyzed data of B domain-deleted hFVIII transgenic animals.

Fig.4A The structure of αLA-CN-hFVIII(△B)-bGHpA fusion genes are shown and two pairs of primer sets for PCR screening, 273-bp for the 5'-end franking region and 751-bp for the B-domain flanking region, respectively. It also shows the genomic digested enzymes and predicted patterns in Southern blot assay.

Fig.4B Rapid screening positive transgenic founders by PCR amplification. NC represented a normal mouse DNA as a negative control. PC represented a hFVIII(△B) plasmid DNA as a positive control.

Fig.4C Southern blot hybridization analysis of transgenic goat (Tg-3431)

harboring B-domain-deleted hFVIII fusion gene. Normal and transgenic goat genomic DNAs were digested with HindIII, PvuII, and XbaI, individually. Arrowheads represented the off-size bands in transgenic goat genome.

Fig.5 confirms the germ-line transmission of transgenic animals carrying hFVIII(△B) exogenic DNA in their F1 generation by using PCR detection and direct PCR product sequencing. The sequencing result exhibited the intact transgenic hFVIII(△B) fragment was acturally present in their F1 offspring's genome.

Fig.6A~6B shows the mammary-specific expression cassette regulated a temporal and spatial expression of exogenous hFVIII RNA in transgenic animals.

Fig.6A Tissue-specific expression of α LA-FVIII transgene by RT-PCR detection. Tissue was removed from lactating mice at 7 days post partum. RNA was isolated from lactating transgenic mouse mammary gland (Ma), heart (H), liver (L), lung (Lu), muscle (M), brain (B), prancreas (P), and in male transgenic mouse mammary fat pad (F). A b-actin primer set was used as internal control.

Fig.6B Stage-specific expression of α LA-FVIII transgene during pre-partum (D-3), lactation (D1 to D22), and weaning (D29 to D36). A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer set was used as an internal control. The ratios of hFVIII/ GAPDH band intensities were plotted by densitometer with computer analysis (Bio-Rad video densitomer, model 620).

Fig.7A~7B shows the Western blot analysis of recombinant hFVIII protein secreted from mammary glands of transgenic mice at different lactating stage.

Fig.7A The secretion of rFVIII protein in milks was collected, 10- to 20-fold diluted and subjected to western blotting with rabbit anti-hFVIII polyclonal antiserum. Lane 1 contained the milk protein harvested from non-transgenic mice

during lactating stage as a negative control and lane 2 contained HPLC-purified hFVIII heavy chain (200-kDa) as a positive control. The orders for D7, D12, D17, and D22 represented the milk proteins harvested from various lactating stages of transgenic mice.

Fig.7B Western blotting analysis of rFVIII light chain protein with mouse anti-hFVIII monoclonal antibody D2. Lane 1 contained the milk protein harvested from non-transgenic mice during lactating stage as a negative control and lane 2 contained the HPLC-purified hFVIII light chain (80-kDa) as a positive control.

Fig.8 shows the Western blot analysis of recombinant hFVIII protein secreted from mammary glands of transgenic goat (Tg-201) at early lactating stage. The secretion of rFVIII protein in milks was collected in days 3, 11 and 22. The collected milks were diluted in 1- to 3-fold and subjected to Western blotting and hybridization with rabbit anti-hFVIII polyclonal antiserum. Lane 1 contained HPLC-purified hFVIII heavy chain (200-kDa) as a positive control and lane 2 contained the milk protein harvested from non-transgenic mice during lactating stage as a negative control.

Fig.9A~9C shows the functional assay of transgenic hFVIII protein by clotting activity analysis. The coagulant activity of hFVIII was measured by reduction of the activated partial thromboplastin time (aPTT) in a one-stage assay (Over, 1984, Scand J. Haematol. 41: 13-24.).

Fig.9A Human FVIII dilutions equivalent to a 1% to 50% dilution of normal plasma (NPP) were prepared in 50 mM imidazole buffer.

Fig.9B NPP standard curve was performed using aPTT clotting assay.

Fig.9C Control and transgenic goat milks diluted in 50%, 20%, and 10% were incubated in duplicate at 37℃ in hFVIII-deficient plasma, followed by the

automated addition of aPTT reagent. The clot time, in seconds, was recorded on an ST2 Coagulometer (Stago).

BRIEF DESCRIPTION OF THE DRAWINGS

The invention relates to transgenic milk that can be used to produce therapeutical recombinant hFVIII for hemophilia patent treatment.

Using mammary gland-specific promoters, a wide range of proteins of bio-pharmaceutical interest have been expressed in rodents, pigs, and dairy animals (Echeland, 1996, Current Opinion in Biotechnology 7: 536; Houdebine et al., 2000, Transgenic Research 9:505). An expression vector, comprising a gene encoding the target protein of interest fused to a milk promoter gene, is introduced by microinjection into the pronucleus of a one-cell embryo. Upon germ line integration and expression, the transgene becomes a dominant Mendelian genetic characteristic that is inherited by the progeny of the founder animal. Mammalian mammary epithelial cells have the capacity to carry out complex protein synthesis with a variety of posttranslational modifications and folding. The milk of transgenic livestock presents a starting material from which human diagnostic or pharmaceutically-active and therapeutic proteins may be purified using established technologies.

Milk Specific Promoters

A variety of transcriptional promoters that preferentially activate transcription in mammary epithelial cells are available. These include the promoters that control the genes encoding milk proteins such as caseins (α S1-, α S2-, β-, γ-, and κ -casein), beta-lactoglobulin (Clark et al., 1989,

Bio/Technology 7: 487-492), whey acid protein (Gordon et al., 1987, Bio/Technology 5: 1183-1187), and alpha-lactalbumin (Soulier et al., 1992, FEBS Letters 297: 13). Casein promoters may be derived from the alpha, beta, gamma or kappa casein genes of any mammalian species; a preferred promoter is derived from the goat beta casein gene (DiTullio, 1992, Bio/Technology 10:74-77).

The DNA sequences of many these promoters are available, e.g., in GenBank and in scientific publications such as 1) rat alpha-lactalbumin (Richards et al., 1981, J. Biol. Chem. 256: 526-532); 2) rat WAP (Campbell et al., 1984, Nucleic Acids Res. 12: 8685-8697); 3) rat alpha-casein (Jones et al., 1985, J. Biol. Chem. 260: 7042-7050); 4) rat alpha-casein (Lee and Rosen, 1983, J. Biol. Chem. 258: 10794-10804); 5) human alpha-lactalbumin (Hall, 1987, Biochem. J. 242: 735-742); 6) bovine alpha-S1 casein (Stewart, 1984, Nucleic Acids Res. 12: 389); 7) bovine alpha-casein (Gorodetsky et al., 1988, Gene 66: 87-96); 8) bovine alpha-casein (Alexander et al., 1988, Eur. J. Biochem. 178: 395-401); 9) bovine alpha-S2 casein (Brignon et al., 1977, FEBS Letters 188: 48-55); 10) bovine alpha-lactoglobulin (Jamieson et al., 1987, Gene 61: 85-90; Alexander et al., 1989, Nucleic Acids Res. 17: 6739). For additional regulatory control or stringency, other regulatory sequences such as adjacent sequences, can be obtained from these genes or from homologous genes of other mammals using these promoter sequence as probes to screen genomic libraries.

Signal Peptide Sequences

The recombinant gene construction can also include a signal sequences, particularly a signal peptide sequences of a milk specific gene. For the best secretion efficiency of forigen protein in the mammary gland, the milk-specific signal peptide sequence can be the secretional signal sequence which naturally

occurs with the selected milk-specific promoter used in the construct, which are described below. For example, signal sequences from genes coding for caseins, e.g., alpha, beta, gamma or kappa caseins, beta lactoglobulin, whey acid protein, and lactalbumin can be used.

Transgenesis Constructions

A 2.0-kb promoter sequence of bovine alpha-lactalbumin (α LA) was generated by PCR amplification using a genomic DNA from high milk-producing Holstein cow, which obtained from National Taiwan University Farm, as the template. This PCR product containing entire α LA promoter and 19-aa leader sequence (SEQ ID: No. 1) or replacment with bovine α S1-casein 15-aa signal peptide sequence (SEQ ID: No.2) which created a restriction enzyme, HpaI, cloning site in the downstream sequence. These two types of promoter and signal peptide sequences were then subsequently inserted into the pCR3 vector (Invitrogene, San Diego, CA).

For the full-length hFVIII construction, the resulting 7.0-kb plasmid containing α LA promoter and its intact signal peptide sequence was double digested with MluI and PstI and treated with calf intestinal phosphatase (Boehringer Mannheim, Indianapolis, IN). The pCMV5/hFVIII plasmid containing the intact human FVIII coding sequence was also double digested with MluI and PstI. The in frame sequence from the α LA leader peptide through the hFVIII junction was determined using the Dye Terminator sequencing system (Applied Biosystems Inc., Foster, CA). The 9.7-kb transgene consisting of 2.0-kb bovine α LA promoter, 7.2-kb hFVIII cDNA, and 0.5-kb bovine GH gene polyadenosine signal sequence was separated from plasmid pCR-α LA/hFVIII-5 using ClaI and XbaI digestion and purified for microinjection on the twice $CsCl_2$

gradient ultra-centrifugation.

For the B domain-deleted hFVIII construction, the resulting 7.0-kb plasmid containing α LA promoter and α S1-casein signal peptide sequence was double digested with HpaI and XhoI and treated with calf intestinal phosphatase (Boehringer Mannheim, Indianapolis, IN). The pCMV5/hFVIII plasmid containing the intact human FVIII coding sequence was used as template to generate a 2233-bp rhFVIII A-domain fragment using degenerate PCR, phFVIII-HpaI(+): 5'-GGT TAA CTG CCA CCA GAA GAT A-3' (SEQ ID: NO.3) and phFVIII-741aa(-): 5'-AAG CTT CTT GGT TCA ATG GC-3' (SEQ ID: NO.4), and a 2085-bp rhFVIII C-domain fragment amplified by phFVIII-1643aa(+): 5'-AAG CTT GAA ACG CCA TCA ACG GGA A-3' (SEQ ID: NO.5) and phFVIII-XhoI(-): 5'-CTC GAG CCT CAG TAG AGG TCC TGT-3' (SEQ ID: NO.6), respectively. Equal molar ratio of A-domain segment, C-domain segment and pCR3-α LA vector were co-ligated and transformed into host competent cells. The in frame sequence from the α S1-casein leader peptide through the hFVIII junction was determined using the Dye Terminator sequencing system (Applied Biosystems Inc., Foster, CA). The 6.8-kb transgene consisting of 2.0-kb bovine α LA promoter and aS1-casein leader sequence, 4.3-kb hFVIII cDNA, and 0.5-kb bovine GH gene polyadenosine signal sequence was separated from plasmid pCR-α LA/hFVIII($\triangle$B) using ClaI and XbaI digestion and purified for microinjection on the twice $CsCl_2$ gradient ultra-centrifugation.

Transgenic Animal Productions

The purified transgene was microinjected into the male pronuclei of fertilized eggs from superovulated female mice of the outbreed ICR strain and transferred to recipient pseudo-pregnant females as previously described (Chen et

al., Transgenic Res 4: 52-59; 1995). For transgenic goat production, the pronuclear stage embryos were flushed from the donor goat's oviduct at the one and half day after insemination by means of surgical method. In order to obtain higher embryo numbers, every embryo-donating goat had been treated with endocrine so as to achieve the object of superovulation. Such a superovulation treatment comprised of administrating intramuscular of follicular stimulating hormone (FSH) to the embryo-donating goat sequentially for 4 days since eighth day of estrous cycle twice a day with interval of 12 hours and the dosage was decreased daily as 4-, 3-, 2-, and 1-mg, respectively. As the first dosage at the third day, co-administrated with 1000iu human chorionic gonadotropin (HCG) which resulted in detection of estrous after 54 hours whereupon gave two artificial insemination (AI) with a interval of 12 hours. Then, at about one and half days after conception, the goat one-cell stage embryos were collected with a sterile glass capillary tube via surgical embryo flushing method. The collected embryo was transferred into another petri dish where it was rinsed more than ten times. Thereafter, it was placed under a phase contrast microscope at 400X amplification for gene microinjection manipulation. After a transient in vitro culture, the healthly microinjected embryos were then transferred into recipient oviducts for further conceptus development.

Recombinant hFVIII Transgene Determination

The resulting pups were rapidly screened for the transgene by PCR amplification of tail or ear tissue DNA. PCR was performed using one set of primer, pαLA-124(+): 5'-CTC TCT TGT CAT CCT CTT CC-3' (SEQ ID: NO.7) and phFVIII-149(-): 5'-GGT TAC GCG TCA AGA TTC TGA-3' (SEQ ID: NO.8), which defined a 273-bp region spanning the αLA promoter, secretion signal

sequence and hFVIII cDNA junctional sequence. For the B domain-deleted hFVIII transgene detection, additional primer pairs was designed, phFVIII-ACJ(+) : 5'-AGA CTT TCG GAA CAG AGG CA-3' (SEQ ID: NO.9) and phFVIII-ACJ(-): 5'-ATC TTT TTC CAG GTC AAC ATC A-3' (SEQ ID: NO.10), which defined a 751-bp region flanking A-C recombinant junction. The positive PCR screening results for transgenic animals were further confirmed by Southern blot analysis. Ten micrograms of genomic DNA were individurally digested with BamHI, HindIII, PvuII, and XbaI restriction enzymes at 37℃ overnight, electrophoresed on a 0.8% agarose gel, and transferred to a Durose membrane (Stratagene, La Jolla, CA). A KpnI fragment of hFVIII-specific cDNA (1.8-kb) was used as a radioactive probe to hybridize the membrane. Blots were subjected to autoradiography for three days at -20℃.

Recombinant hFVIII RNA Expression in Transgenic Animals

The temporal and spatial expression of hFVIII RNA in transgenic animals was analyzed using a reverse transcription-polymerase chain reaction (RT-PCR). Total RNA from different tissues including the heart, liver, lung, muscle, mammary gland, brain, pancrease, and kidney of female transgenic mice during lactation periods (Day 1 to Day 28 post partum) was extracted using the acid guanidinium thiocyanate method (Chomczynski and Sacchi, 1987, Anal. Biochem. 162: 156-159). One microgram of total RNA was treated twice with 10 units of DNase I (Gibco BRL, Gaithersburg, MD) and phenol-chloroform extracted. RNA pellets were resuspended in 15 μl DEPC-water and then used to synthesize the first-strand cDNA with random primers and SuperScript reverse transcriptase (Gibco BRL, Gaithersburg, MD) in a total volume of 25 ml. The reaction was carried out at 42℃ for 1 hr.

For further PCR amplification, an aliquot (1/10) of the RT product was adjusted to contain 0.1 μg of each primer and additional buffer was added for a total volume of 50 μl. PCR was performed for 30 cycles (94℃, 1 min; 55 ℃, 2 min; 72℃, 2 min). The primers used included a pair of hFVIII-specific primers, phFVIII-F2(+):5'-CAT TCT ATT CAT TTC AGT GGA CA-3' (SEQ ID: NO.11) and phFVIII-R2(-): 5'-GAG ATG TAG AGG CTG GAG AAC T-3' (SEQ ID: NO.12), and a pair of glyceraldehydes-3-phosphate dehydrogenase (GAPDH), as well as a pair of β-actin. Both β-actin and GAPDH are the universal transcripts in every cell and were used as the internal controls of RT-PCR.

Immunoblot Analysis of Transgenic Milk Proteins

Milk was collected from lactating females as previously described (Simons et al., 1987, Nature 328: 530-532.) and analyzed using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Cheng et al., 1998, Human Gene Therapy 9: 1995-2003). Ten- to twenty-fold dilutions of the collected milk, in 75 mM Tris-HCl buffer at pH 6.8, from different lactation periods were diluted in SDS-PAGE sample buffer with 5% 2-mercaptoethanol and electrophoresed on 7.5% gel. To estimate production levels, HPLC-purified recombinant hFVIII standard was diluted to 10 μg/ml in normal mouse milk and electrophoresed alongside the transgenic milk samples. Proteins were electrotransferred from the gel to a PVDF membrane (NEN Life Science Products, Boston, MA). The blots were probed with primary antibodies recognizing hFVIII at 2 to 10 μg/ml and washed with phosphate-buffered saline containing 0.1% Tween-20 (PBS-T). Blots were reacted with horseradish peroxidase (HRP)-conjugated second antibodies at 0.2 mg/ml. Polyclone antibody C6 recognize the heavy chain of hFVIII rang from 80-200 kDa and monoclone antibody D2 recognize the light chain of hFVIII at 75-80 kDa.

The blot was then developed with the chemiluminescent ECLTM detection system (Amersham, UK) and exposure to x-ray film. Band intensities were compared by densitometry.

Secretion of hFVIII protein from the transgenic milk was determined quantitatively with the enzyme-linked immunosorbent assay (ELISA) as previously described (Chen et al., 1993, J Virol 67: 2142-2148). Briefly, plates were coated with capture antibodies (ESH-5 and ESH-8, 500ng each per well; American Diagnostics, Greenwich, CT) in carbonate buffer and incubated for 1 hour at 37 ℃. Plates were washed with 0.05% Tween-20 in phosphate-buffered saline (PBS) and blocked in 50 mM Tris (pH 7.2), 150 mM NaCl, 0.5% gelatin, and 0.05% Tween-20 for 2 hour at 37 ℃. Purified recombinant FVIII (Hyland, Baxter Healthcare, CA), prepared in blocking buffer with 1:50 normal murine milk, served as the standard. Samples and standards were incubated for 1 hour at 37℃. Detection antibody (rabbit anti-human FVIII 1: 10,000 dilution) was added and allowed to react with hFVIII at 37℃ for 1 h. After removing the first antibody using four washes with PBS, a second antibody, goat anti-rabbit immunoglobulin antibody conjugated with horseradish peroxidase, was added (1: 3,000 dilution) for a further 1 h of incubation. The plates were washed again thoroughly with PBS, and 100 μ l of substrate solution (2 mg of 0-phenylenediamine dissolve in 1 ml of 1M phosphate citrate plus 0.02% H_2O_2) was added for development. After 30 min, 100 μl of 1M H_2SO_4 was added immediately to stop the reaction and the colors were measured by optical density at 492 nm.

The Feature of α-Lactalbuimn Mammary Expression Cassette

Expression of rFVIII protein in the milk of transgenic animals driven by

bovine α-lactalbuimn regulatory sequence seems more efficient than the other transgene constructions, which were controlled by ovine β-lactoglobulin promoter (Niemann et al., 1999, Transgenic Res 8: 237-247.) as well as mouse WAP regulatory sequence (Paleyanda et al., 1997, Nature Biotech 15: 971-975). Expression of the bovine αLA gene has been shown to be the most lactation-specific of all bovine milk protein genes (Goodman and Schanbacher, 1991, Biochem Biophys Res Commun 180:75-84). Use of the αLA 19-aa secretary peptide (SEQ ID: NO: 13) or αS1-casein 15-aa signal peptide (SEQ ID: NO: 14) to lead the rFVIII protein secretion and the bGH polyadenosylation signal to stabilize the steady-state of hFVIII RNA molecules, which may also have contributed to our success. Actually, there are several previous reports demonstrated that mammary specific transgenes driven by αLA promoter have resulted in high levels of gene expression up to 3.7 mg/ml (Hochi et al., 1992, Mol Reprod Dev 33: 160-164; Soulier et al., 1992, Fed Eur Biol Soc Lett 297: 13-20; Maschio et al., 1991, Biochem J 275:459-465) in their milks. This characteristic of the bovine αLA gene makes its regulatory elements potentially useful as a mammary expression system in transgenic animals. In contrast to the caseins and b-lactoglobulin, the production of αLA mRNA increases suddenly at paturition, remains elevated during lactation, and drops sharply at dry-off and during involution. The rFVIII protein profile lead by the αLA promoter and 19-aa secretion signal peptide or αS1-casein 15-aa signal peptide follows a similar trend. The lactation specificity of the regulatory regions used to control mammary expression in transgenic animals may be important when foreign proteins that have biological activities are expressed because these proteins may exhibit their biological function in the animals if they are secreted before tight junctions of

mammary epithelial cells are formed.

The Feature of hFVIII Protein Expression

Clotting factor IX (FIX), adenosine deaminase (ADA), and other cDNAs were expressed at high levels from retroviral vectors in rat fibroblasts (Miller, 1992, Nature 357: 455-460; Palmer et al., 1991, Proc Natl Acad Sci USA 88: 1330-13334), whereas the FVIII cDNA was expressed at very low levels from these vectors in primary human fibroblast cells (Hoeben et al., 1990, J Biol Chem 265: 7318-7323; Lynch et al., 1993, Hum Gene Ther 4: 259-272). It has been found that FVIII RNA steady-state levels were reduced 100-fold from a FVIII retroviral vector compared to the same vector expressing other cDNAs, and FVIII vector titers were correspondingly reduced 100-fold compared to other vectors (Lynch et -al., 1993, Hum Gene Ther 4: 259-272). A large part of the inhibitory effect of the FVIII cDNA on expression from a retroviral vector has been localized to a 1.2-kb fragment, derived from the A2 and A3 domains, which decreased the steady-state RNA levels 100- to 200-fold and decreased vector titers 10-fold (Koeberl et al., 1995, Hum Gene Ther 6: 469-479). The mechanism by which the FVIII cDNA inhibitory sequence (INS) decreases RNA and protein expression from expression vectors remain to be established. Recently, experimental data provided ample evidence that the human FVIII cDNA contains elements that repress its own expression at the level of transcription (Hoeben et al., 1995, Blood 85: 2447-2454). Fallaux and coworkers (1996, Mol Cell Biol 16: 4264-4272) identified a 305-bp region derived from exons 9 to 11 that encodes a nuclear-matrix attachment region (MAR), also called the scaffold-attached region (SAR). Yeast MARs elements can play an important role in transcriptional silencing (Newlon and Theis, 1993, Transgenic Res 8: 237-247). The B domain

can be deleted without any significant effect on specific procoagulant activity (Pittman et al., 1993, Blood 81:2925-2935; Chao et al., 2000, Blood, 95: 1594-1599). B-domain-deleted hFVIII cDNA (BDD-hFVIII) is 4.3 kb and primary truncate polypeptide (hFVIII△B) is 1448 aa (SEQ ID: NO.15) not thought feasible for testing in transgenic animals previously.

The present invention is further depicted in the illustration of examples, but the descriptions made in the examples should not be construed as a limitation on the actual application of the present invention.

EXAMPLE 1

Generations of transgenic mice, pigs and goats carrying the full-length hFVIII gene in mammary-specific expression cassette

Out of 79 potential transgenic founder mice, 17 were identified as being transgenic by the PCR screening (Fig. 1B). Two transgenic founder goats carrying the full-length hFIII also have been identified by PCR screening (Fig. 1C) as well as two lines of transgenic pigs have been established. To quantify the transgene copy numbers and to understand the integration patterns of the foreign gene within the genome the transgenic animals, restriction enzyme BamHI was used to digest the genomic DNA, cut once within the transgene, and subjected to Southern blotting analysis. On hybridization using the 1.8-kb KpnI fragment of human-specific FVIII sequence as a probe (Fig. 1A), a 9.7-kb band representing monomeric transgene copies present in head-to-tail joining (H-T) tandem repeats was observed in all cases. A 4.6-kb hybridization band was observed in the blots of all transgenic mice present in tail-to-tail joining (T-T) inverted repeats (Fig. 1D). Different intensities of the hybridization signal were also observed indicating

the presence of different transgene copy numbers in these mice. Further detail quantification using slot-blot hybridization (Fig. 2A~2B), they could be distinguished into three groups according to the copies of transgene integration. For low copy number (defined as 1-5 copies per cell), there were six transgenic lines including αLAF-7, -15, -33, -36, -43 and -54. Transgenic founders carrying middle copy number (10-20 copies per cell) were found in nine lines including αLAF-9, -13, -18, -27, -28, -29, -30, -38 and -39. In addition, there were two lines (αLAF-8 and -25) carrying high copy number (40-50 copies per cell) of hFVIII transgene in their genome. All of the transgenic mice harboring αLA-hFVIII DNAs presented two-to-three off-size bands (Fig. 1D) besides the predicted transgene junctions. These bands were probably rearranged transgene sequences, some of which were integration sites representing transgene-cellular junctions.

Breeding lines were established from all transgenic founder animals by crossing them with normal ICR mice, Alpine goats (Fig. 1C), or hybride pigs. The multiple integrated copies of the transgene were found to be stably germ-line transmitted among twelve (7 females and 5 males) out of the 17 transgenic founders.

EXAMPLE 2

Generations of transgenic mice and goats carrying the B-domain deleted hFVIII gene in mammary-specific expression cassette

B-domain deleted hFVIII transgene construct driven by αLA promoter and αS1-casein secretary peptide sequence was shown in Fig. 3A. In N-terminal of rhFVIII polypeptide, a 15-aa αS1-casein signal peptide was added for leading newly synthesized rhFVIII secretion into mammary ductal cavity (Fig. 3B). In the

middle of rFVIII polypeptide, the A-domain (741-residue) has been created to join the C-domain (1643-residue) for completely deleted the B-domain segment (Fig. 3C).

Out of 65 potential transgenic founder mice, 15 were identified as being transgenic by the two sets of primers, one pair of primer located in 5'-promoter junction, the other pair of primer flanking the B-domain deletion region (Fig. 4A). The PCR screening was shown in Fig. 4B. To quantify the transgene copy numbers and to understand the integration patterns of the foreign gene within the genome of the transgenic mice, restriction enzyme HindIII, PvuII, and XbaI were used to digest the genomic DNA, cut once within the transgene, and subjected to Southern blotting analysis (Fig. 4C). The result showed that transgenic goat (Tg-3431) harboring B-domain-deleted hFVIII fusion gene clearly exhibited one to two off-size bands in their genome when compairing with normal goat genome.

Germ-line transmission ability of transgenic animals carrying hFVIII(△B) exogenic DNA in their F1 generation was confirmed by using PCR detection and direct PCR product sequencing. The sequencing result exhibited the transgenic hFVIII(△B) fragment was acturally present in their F1 offspring's genome of transgenic goats (Fig. 5).

EXAMPLE 3

Temporal and spatial expression of exogenous hFVIII RNA in transgenic animals driven by mammary-specific expression cassette

To characterize the spatial expression of the new developed mammary-specific expression cassette containing hFVIII in this invention, total tissue RNAs were extracted from different organs in the breeding lines established from the

transgenic founder mice. Reverse-transcriptional polymerase chain reaction (RT-PCR) analysis was performed using two pair of primer sets, one was phFVIII-F2(+) (SEQ ID: NO.11) and phFVIII-R2(-) (SEQ ID: NO.12) specific to human FVIII cDNA franking 3'-coding region, the other was b-actin primer set as an internal control. As shown in Fig. 6A, the transgene expressed transcriptant of the 410-bp hFVIII RT-PCR product was found in the mammary gland of lactating transgenic mouse. No homologous transcripts were detectable in the heart (H), liver (L), lung (LU), muscle (M), brain (B), prancreas (P), or in male transgenic mouse mammary fat pads (F). All examined tissues showed a 420-bp amplified fragment for mouse b-actin to assess the integrity and quantity of RNA in each sample. The efficiency of DNase I treatment to eliminate DNA contamination was determined using RNA from a transgenic mammary gland. When the reverse transcriptase was omitted from the reaction, no amplification was observed (Fig. 6A, last lane).

Temporal expression studies were performed on the F1 transgenic mice in the breeding lines established from founder mice αLAF-15 and -18. Total RNAs were extracted at different physiological states in the transgenic mammary gland using tissue biopsies subjected to RT-PCR detection. The results showed that the recombinant hFVIII mRNA was expressed in all lactating periods (D1, D8, D15 and D22, shown in Fig. 6B), whereas the same transcripts were not detectable in the later stages of pregnancy before parturition (D-3) and in the weaning stage after lactation (D29 and D36, in Fig. 6B). The 2.0-kb bovine αLA promoter had a highly tissue-specific and stage-specific gene regulation activity in the mammary epithelial cells during the lactation period.

To quantify the level of hFVIII mRNA in the mammary glands of

transgenic mice, densitometric analysis was performed, comparing the intensity of the 250-bp band in each lane normalized to the level of signal generated upon co-amplification of the mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers. The intensity of the hFVIII transcript increased eight-fold and ten-fold upon Day 8 and Day 15 post-parturition, respectively, while a two-fold decrease was present on Day 22 of the later lactation (Fig. 6B), compared to Day 1 of the newly lactating stage.

EXAMPLE 4

Expression and secretion of recombinant hFVIII proteins into the transgenic milks

To determine the production of recombinant hFVIII and to compare the secretion efficiency of rFVIII among the different transgenic mouse lines, milk was collected from five lines of F0, F1 and F2 females during lactation. Proteins from both non-transgenic control and transgenic skim milks were separated in SDS-PAGE and followed by Western blot analysis (Fig. 7A~7B). No polypeptides characteristic of recombinant hFVIII were detected in control mouse milk using the hFVIII-specific polyclonal antibody. In transgenic milk, rFVIII was detected as a heterogenous group of polypeptides of approximately 80-200 kDa, of which the 92-200 kDa polypeptides represent the heavy chain and its proteolytic cleavage products or different glycosylational modification patterns. The small molecule of an 80 kDa corresponding to the light chain containing hFVIII C2 domain was also detected by this antibody (Fig. 7A). In similar experiments using monoclonal antibodies that recognize specific hFVIII light chain C2 domain structures, a specific polypeptide corresponding in size to the 80

kDa was observed as compared with the HPLC-purified hFVIII C2 domain peptide (Fig. 7B).

In the transgenic goat (Tg-201), milk was collected from days 3, 11 and 22 during early lactation stage for Western blot assay (Fig. 8). No polypeptides characteristic of recombinant hFVIII were detected in control goat milk using the hFVIII-specific polyclonal antibody. In transgenic milk, rFVIII was detected as a heterogenous group of polypeptides of approximately 80-200 kDa, which represent the heavy chain and its proteolytic cleavage products or different glycosylational modification patterns.

EXAMPLE 5

Biological function assay for clotting activity of recombinant hFVIII derived from transgenic milks

The coagulant activity of hFVIII was measured by reduction of the activated partial thromboplastin time (APTT) in a one-stage assay (Over, 1984, Scand J. Haematol. 41: 13-24.). Human FVIII dilutions equivalent to a 1:10 to 1:120 dilution of normal plasma were prepared in 50 mM imidazole buffer, pH 7.4, containing 100mM sodium choride, 0.1% BSA and 0.01% Tween-20. Control and transgenic mouse milks diluted 1:20, 1:30, and 1:40 were incubated in duplicate at 37 ℃ in hFVIII-deficient plasma (Sigma Diagnostics, St. Louis, MO), followed by the automated addition of APTT reagent (Dade Behring, Marburg, Germany). Clot formation was initiated by the addition of 35 mM $CaCl_2$. The clot time, in seconds, was recorded on an ST2 Coagulometer (Stago). Semi-logarithmic analysis was used to plot the clot time versus amount of FVIII. Control mouse milk gave results similar to the reagent blank.

The concentrations of rFVIII ranged from 7.0 to 50.2 μg/ml, over 35- to 200-fold higher than that in normal human plasma (Table 1). The expression levels of rFVIII from three groups on different line carrying low, middle and high copy numbers of transgenes were not significantly different. Recombinant hFVIII production generally tended to increase through the lactation period, but dropped dramatically at the end of lactation. This result was in parallel with the RNA transcript assay (Fig. 6A~6B) as well as the typical mouse milk production curve, mouse milk yield increased throughout lactation, peaked approximately D15 of lactation, and then decreased dramatically after D20.

The functional activity of rFVIII was examined using one-stage clotting assays (Table 1) and this approach demonstrated that rFVIII secreted in the milk of transgenic mice was biologically active. In the clotting assay, the addition of transgenic mouse milk, but not control milk, to FVIII-deficient human plasma resulted in the restoration of normal coagulant activity. Up to 13.41 U/ml of rFVIII procoagulant activity was detected. The activity of rFVIII was detected throughout lactation as shown in Table 1. The same result for rFVIII clotting activity derived from transgenic goat also has been demonstrated and showed in Fig. 9A~9C.

Table 1: Recombinant hFVIII concentration and coagulation activity in the milk of transgenic animals with different groups of transgene copy, lines and generations

No. of transgenic rFVIII concentration mice	Lactations and days (L/D)	Coagulation activity assay (U/ml)[a]	(μg/ml)
Low copy group(1-5 copies)			
aLAF15 (Fo)	L1/D7	4.82 ± 0.43	22.84 ± 1.65
(n=1)	L1/D12	7.11 ± 0.76	30.58 ± 1.37
	L1/D19	3.60 ± 0.51	19.87 ± 0.96
aLAF36 (F1)	L1/D7	6.38 ± 1.85	29.26 ± 3.58
(n=4)	L1/D17	5.64 ± 2.66	24.92 ± 3.04
	L2/D7	13.41 ± 2.87	50.21 ± 5.73
	L2/D17	9.46 ± 2.04	40.84 ± 4.91
Middle copy group(10-20 copies)			
aLAF18 (F1)	L1/D7	0.98 ± 0.39	7.03 ± 2.56
(n=3)	L1/D12	6.14 ± 0.98	32.07 ± 4.30
	L1/D17	8.91 ± 2.15	41.37 ± 7.78
aLAF28 (F2)	L1/D7	3.01 ± 0.95	9.23 ± 3.74
(n=3)	L1/D12	6.55 ± 2.07	35.56 ± 6.08
	L2/D17	7.81 ± 2.53	38.11 ± 5.72
High copy group(40-50copies)			
aLAF25 (F1)	L1/D8	11.28 ± 2.33	46.33 ± 4.57
(n=3)	L1/D15	8.72 ± 1.79	32.92 ± 3.97
	L1/D21	1.56 ± 0.24	15.08 ± 4.84
aLAF25 (F2)	L1/D8	7.92 ± 2.01	39.29 ± 6.89
(n=3)	L1/D15	8.58 ± 2.78	43.48 ± 5.21
	L1/D21	1.22 ± 0.61	13.32 ± 5.05

[a] One unit of rFVIII was defined as equivalent to the amount of human FVIII normally present in 1ml of plasma, approximately 200 ng. Results presented are the average of two independent assays

THE ADVANTAGES OF THE INVENTION

1. The new developed mammary-specific expression cassette containing three important parts:

   (1) 2.0-kb bovine α LA gene promoter which has been shown to be the most lactation-specific of all bovine milk protein genes;

   (2) use of the α S1-CN 15-aa or α LA 19-aa as a secretary peptide to lead the rFVIII protein secretion; and

   (3) the bGH polyadenosylation signal to stabilize the steady-state of hFVIII RNA molecules, which can guaranty the highly expression and efficiently secretion of any exogenic proteins.

2. Transgenic expression delivers the advantages of mammalian cells such as a sophisticated refolding machinery and glycosylation.

3. Transgenic productions are scale-up flexibility since the herd size can be increased rapidly and inexpensively, as well as the relative low-cost and low complexity of the raw product manufacturing facilities as compared to more traditional cell culture facilities.

4. Biological active recombinant hFVIII protein derived from transgenic milks is easily collected by diary automatic milk collection system and simply purified procedure to obtain the massive recombinant proteins.

5. Transgenic mice, goats and pigs expressing high levels of rFVIII either in full-length gene constructs or in B domain-deleted hFVIII will be a great value system for the mass production of biologically active rFVIII in transgenic farm animals.

SEQUENCE LISTING

(1)GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 15

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 63 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLCULE TYPE: artificial synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATGATGTCCT TTGTCTCTCT GCTCCTGGTA GGCATCCTAT TCCATGCCAC CCAGGCTGTT AAC 63

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 50 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLCULE TYPE: artificial synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

ATGAAACTTC TCATCCTTAC CTGTCTTGTG GCTGTTGCTG CCAGGTTAAC 50

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLCULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GGTTAACTGC CACCAGAAGA TA 22

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLCULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

AAGCTTCTTG GTTCAATGGC 20

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLCULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

AAGCTTGAAA CGCCATCAAC GGGAA 25

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLCULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CTCGAGCCTC AGTAGAGGTC CTGT 24

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLCULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CTCTCTTGTC ATCCTCTTCC 20

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleotides

(E) TYPE: nucleic acid

(F) STRANDEDNESS: single

(G) TOPOLOGY: linear

(ii) MOLCULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GGTTACGCGT CAAGATTCTG A 21

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLCULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

AGACTTTCGG AACAGAGGCA 20

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLCULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

ATCTTTTTCC AGGTCAACAT CA 22

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLCULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CATTCTATTC ATTTCAGTGG ACA 23

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLCULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GAGATGTAGA GGCTGGAGAA CT 22

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 amino acids

(B) TYPE: Amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLCULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Met	Met	Ser	Phe	Val	Ser	Leu	Leu	Leu	Val	Gly	Ile	Leu	Phe	His
1				5					10					15

Ala Thr Glu Ala

(3) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(E) LENGTH: 15 amino acids

(F) TYPE: Amino acid

(G) STRANDEDNESS: single

(H) TOPOLOGY: linear

(ii) MOLCULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

Met	Lys	Leu	Leu	Ile	Leu	Thr	Cys	Leu	Val	Ala	Val	Ala	Ala	Arg
1				5					10					15

(3)INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(I) LENGTH: 1448 amino acids

(J) TYPE: Amino acid

(K) STRANDEDNESS: single

(L) TOPOLOGY: linear

(ii) MOLCULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

Met	Lys	Leu	Leu	Ile	Leu	Thr	Cys	Leu	Val	Ala	Val	Ala	Ala	Arg
1				5					10					15
Leu	Thr	Ala	Thr	Arg	Arg	Tyr	Tyr	Leu	Gly	Ala	Val	Glu	Leu	Ser
				20					25					30

Trp	Asp	Tyr	Met	Gln	Ser	Asp	Leu	Gly	Glu	Leu	Pro	Val	Asp	Ala	
				35					40					45	
Arg	Phe	Pro	Pro	Arg	Val	Pro	Lys	Ser	Phe	Pro	Phe	Asn	Thr	Ser	
				50					55					60	
Val	Val	Tyr	Lys	Lys	Thr	Leu	Phe	Val	Glu	Phe	Thr	Asp	His	Leu	
				65					70					75	
Phe	Asn	Ile	Ala	Lys	Pro	Arg	Pro	Pro	Trp	Met	Gly	Leu	Leu	Gly	
				80					85					90	
Pro	Thr	Ile	Gln	Ala	Glu	Val	Tyr	Asp	Thr	Val	Val	Ile	Thr	Leu	
				95					100					105	
Lys	Asn	Met	Ala	Ser	His	Pro	Val	Ser	Leu	His	Ala	Val	Gly	Val	
				110					115					120	
Ser	Tyr	Trp	Lys	Ala	Ser	Glu	Gly	Ala	Glu	Tyr	Asp	Asp	Gln	Thr	
				125					130					135	
Ser	Gln	Arg	Glu	Lys	Glu	Asp	Asp	Lys	Val	Phe	Pro	Gly	Gly	Ser	
				140					145					150	
His	Thr	Tyr	Val	Trp	Gln	Val	Leu	Lys	Glu	Asn	Gly	Pro	Met	Ala	
				155					160					165	
Ser	Asp	Pro	Leu	Cys	Leu	Thr	Tyr	Ser	Tyr	Leu	Ser	His	Val	Asp	
				170					175					180	
Leu	Val	Lys	Asp	Leu	Asn	Ser	Gly	Leu	Ile	Gly	Ala	Leu	Leu	Val	
				185					190					195	

Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr Gln Thr Leu His
200 205 210
Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly Lys Ser Trp
215 220 225
His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp Ala Ala
230 235 240
Ser Ala Arg Ala Trp Pro Lys Met His Thr Val Asn Gly Tyr Val
245 250 255
Asn Arg Ser Leu Pro Gly Leu Ile Gly Cys His Arg Lys Ser Val
260 265 270
Tyr Trp His Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser
275 280 285
Ile Phe Leu Glu Gly His Thr Phe Leu Val Arg Asn His Arg Gln
290 295 300
Ala Ser Leu Glu Ile Ser Pro Ile Thr Phe Leu Thr Ala Gln Thr
305 310 315
Leu Leu Met Asp Leu Gly Gln Phe Leu Leu Phe Cys His Ile Ser
320 325 330
Ser His Gln His Asp Gly Met Glu Ala Tyr Val Lys Val Asp Ser
335 340 345
Cys Pro Glu Glu Pro Gln Leu Arg Met Lys Asn Asn Glu Glu Ala
350 355 360

Glu Asp Tyr Asp Asp Asp Leu Thr Asp Ser Glu Met Asp Val Val
365 370 375

Arg Phe Asp Asp Asp Asn Ser Pro Ser Phe Ile Gln Ile Arg Ser
380 385 390

Val Ala Lys Lys His Pro Lys Thr Trp Val His Tyr Ile Ala Ala
395 400 405

Glu Glu Glu Asp Trp Asp Tyr Ala Pro Leu Val Leu Ala Pro Asp
410 415 420

Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn Asn Gly Pro Gln Arg
425 430 435

Ile Gly Arg Lys Tyr Lys Lys Val Arg Phe Met Ala Tyr Thr Asp
440 445 450

Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu Ser Gly Ile
455 460 465

Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu Leu Ile
470 475 480

Ile Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro His
485 490 495

Gly Ile Thr Asp Val Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys
500 505 510

Gly Val Lys His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile
515 520 525

Phe Lys Tyr Lys Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys
530 535 540
Ser Asp Pro Arg Cys Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn
545 550 555
Met Glu Arg Asp Leu Ala Ser Gly Leu Ile Gly Pro Leu Leu Ile
560 565 570
Cys Tyr Lys Glu Ser Val Asp Gln Arg Gly Asn Gln Ile Met Ser
575 580 585
Asp Lys Arg Asn Val Ile Leu Phe Ser Val Phe Asp Glu Asn Arg
590 595 600
Ser Trp Tyr Leu Thr Glu Asn Ile Gln Arg Phe Leu Pro Asn Pro
605 610 615
Ala Gly Val Gln Leu Glu Asp Pro Glu Phe Gln Ala Ser Asn Ile
620 625 630
Met His Ser Ile Asn Gly Tyr Val Phe Asp Ser Leu Gln Leu Ser
635 640 645
Val Cys Leu His Glu Val Ala Tyr Trp Tyr Ile Leu Ser Ile Gly
650 655 660
Ala Gln Thr Asp Phe Leu Ser Val Phe Phe Ser Gly Tyr Thr Phe
665 670 675
Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr Leu Phe Pro Phe
680 685 690

Ser	Gly	Glu	Thr	Val	Phe	Met	Ser	Met	Glu	Asn	Pro	Gly	Leu	Trp
				695					700					705
Ile	Leu	Gly	Cys	His	Asn	Ser	Asp	Phe	Arg	Asn	Arg	Gly	Met	Thr
				710					715					720
Ala	Leu	Leu	Lys	Val	Ser	Ser	Cys	Asp	Lys	Asn	Thr	Gly	Asp	Tyr
				725					730					735
Tyr	Glu	Asp	Ser	Tyr	Glu	Asp	Ile	Ser	Ala	Tyr	Leu	Leu	Ser	Lys
				740					745					750
Asn	Asn	Ala	Ile	Glu	Pro	Arg	Ser	Leu	Lys	Arg	His	Gln	Arg	Glu
				755					760					765
Ile	Thr	Arg	Thr	Thr	Leu	Gln	Ser	Asp	Gln	Glu	Glu	Ile	Asp	Tyr
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Asp	Asp	Thr	Ile	Ser	Val	Glu	Met	Lys	Lys	Glu	Asp	Phe	Asp	Ile
				785					790					795
Tyr	Asp	Glu	Asp	Glu	Asn	Gln	Ser	Pro	Arg	Ser	Phe	Gln	Lys	Lys
				800					805					810
Thr	Arg	His	Tyr	Phe	Ile	Ala	Ala	Val	Glu	Arg	Leu	Trp	Asp	Tyr
				815					820					825
Gly	Met	Ser	Ser	Ser	Pro	His	Val	Leu	Arg	Asn	Arg	Ala	Gln	Ser
				830					835					840
Gly	Ser	Val	Pro	Gln	Phe	Lys	Lys	Val	Val	Phe	Gln	Glu	Phe	Thr
				845					850					855

Asp Gly Ser Phe Thr Gln Pro Leu Tyr Arg Gly Glu Leu Asn Glu
860 865 870

His Leu Gly Leu Leu Gly Pro Tyr Ile Arg Ala Glu Val Glu Asp
875 880 885

Asn Ile Met Val Thr Phe Arg Asn Gln Ala Ser Arg Pro Tyr Ser
890 895 900

Phe Tyr Ser Ser Leu Ile Ser Tyr Glu Glu Asp Gln Arg Gln Gly
905 910 915

Ala Glu Pro Arg Lys Asn Phe Val Lys Pro Asn Glu Thr Lys Thr
920 925 930

Tyr Phe Trp Lys Val Gln His His Met Ala Pro Thr Lys Asp Glu
935 940 945

Phe Asp Cys Lys Ala Trp Ala Tyr Phe Ser Asp Val Asp Leu Glu
950 955 960

Lys Asp Val His Ser Gly Leu Ile Gly Pro Leu Leu Val Cys His
965 970 975

Thr Asn Thr Leu Asn Pro Ala His Gly Arg Gln Val Thr Val Gln
980 985 990

Glu Phe Ala Leu Phe Phe Thr Ile Phe Asp Glu Thr Lys Ser Trp
995 1000 1005

Tyr Phe Thr Glu Asn Met Glu Arg Asn Cys Arg Ala Pro Cys Asn
1010 1015 1020

Ile Gln Met Glu Asp Pro Thr Phe Lys Glu Asn Tyr Arg Phe His
1025 1030 1035
Ala Ile Asn Gly Tyr Ile Met Asp Thr Leu Pro Gly Leu Val Met
1040 1045 1050
Ala Gln Asp Gln Arg Ile Arg Trp Tyr Leu Leu Ser Met Gly Ser
1055 1060 1065
Asn Glu Asn Ile His Ser Ile His Phe Ser Gly His Val Phe Thr
1070 1075 1080
Val Arg Lys Lys Glu Glu Tyr Lys Met Ala Leu Tyr Asn Leu Tyr
1085 1090 1095
Pro Gly Val Phe Glu Thr Val Glu Met Leu Pro Ser Lys Ala Gly
1100 1105 1110
Ile Trp Arg Val Glu Cys Leu Ile Gly Glu His Leu His Ala Gly
1115 1120 1125
Met Ser Thr Leu Phe Leu Val Tyr Ser Asn Lys Cys Gln Thr Pro
1130 1135 1140
Leu Gly Met Ala Ser Gly His Ile Arg Asp Phe Gln Ile Thr Ala
1145 1150 1155
Ser Gly Gln Tyr Gly Gln Trp Ala Pro Lys Leu Ala Arg Leu His
1160 1165 1170
Tyr Ser Gly Ser Ile Asn Ala Trp Ser Thr Lys Glu Pro Phe Ser
1175 1180 1185

Trp Ile Lys Val Asp Leu Leu Ala Pro Met Ile Ile His Gly Ile
1190 1195 1200

Lys Thr Gln Gly Ala Arg Gln Lys Phe Ser Ser Leu Tyr Ile Ser
1205 1210 1215

Gln Phe Ile Ile Met Tyr Ser Leu Asp Gly Lys Lys Trp Gln Thr
1220 1225 1230

Tyr Arg Gly Asn Ser Thr Gly Thr Leu Met Val Phe Phe Gly Asn
1235 1240 1245

Val Asp Ser Ser Gly Ile Lys His Asn Ile Phe Asn Pro Pro Ile
1250 1255 1260

Ile Ala Arg Tyr Ile Arg Leu His Pro Thr His Tyr Ser Ile Arg
1265 1270 1275

Ser Thr Leu Arg Met Glu Leu Met Gly Cys Asp Leu Asn Ser Cys
1280 1285 1290

Ser Met Pro Leu Gly Met Glu Ser Lys Ala Ile Ser Asp Ala Gln
1295 1300 1305

Ile Thr Ala Ser Ser Tyr Phe Thr Asn Met Phe Ala Thr Trp Ser
1310 1315 1320

Pro Ser Lys Ala Arg Leu His Leu Gln Gly Arg Ser Asn Ala Trp
1325 1330 1335

Arg Pro Gln Val Asn Asn Pro Lys Glu Trp Leu Gln Val Asp Phe
1340 1345 1350

Gln Lys Thr Met Lys Val Thr Gly Val Thr Thr Gln Gly Val Lys
1355 1360 1365
Ser Leu Leu Thr Ser Met Tyr Val Lys Glu Phe Leu Ile Ser Ser
1370 1375 1380
Ser Gln Asp Gly His Gln Trp Thr Leu Phe Phe Gln Asn Gly Lys
1385 1390 1395
Val Lys Val Phe Gln Gly Asn Gln Asp Ser Phe Thr Pro Val Val
1400 1405 1410
Asn Ser Leu Asp Pro Pro Leu Leu Thr Arg Tyr Leu Arg Ile His
1415 1420 1425
Pro Gln Ser Trp Val His Gln Ile Ala Leu Arg Met Glu Val Leu
1430 1435 1440
Gly Cys Glu Ala Gln Asp Leu Tyr
1445